

The stimulatory action of amphiphysin on dynamin function is dependent on lipid bilayer curvature

Yumi Yoshida^{1,2,6}, Masahiro Kinuta^{1,6},
Tadashi Abe¹, Shuang Liang¹,
Kenta Araki^{1,2}, Ottavio Cremona³,
Gilbert Di Paolo⁴, Yoshinori Moriyama²,
Tatsuji Yasuda⁵, Pietro De Camilli⁴
and Kohji Takei^{1,*}

¹Department of Neuroscience, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan, ²Department of Biochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan, ³DIBIT-Scientific Institute San Raffaele Università Vita – Salute San Raffaele, Milano, Italy, ⁴Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT, USA and ⁵Department of Cell Chemistry, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan

Amphiphysin is a major dynamin-binding partner at the synapse; however, its function in fission is unclear. Incubation of large unilamellar liposomes with mice brain cytosol led to massive formation of small vesicles, whereas cytosol of amphiphysin 1 knockout mice was much less efficient in this reaction. Vesicle formation from large liposomes by purified dynamin was also strongly enhanced by amphiphysin. In the presence of liposomes, amphiphysin strongly affected dynamin GTPase activity and the recruitment of dynamin to the liposomes, but this activity was highly dependent on liposome size. Deletion from amphiphysin of its central proline-rich stretch dramatically potentiated its effect on dynamin, possibly by relieving an inhibitory intramolecular interaction. These results suggest a model in which maturation of endocytic pits correlates with the oligomerization of dynamin with either amphiphysin or other proteins with similar domain structure. Formation of these complexes is coupled to the activation of dynamin GTPase activity, thus explaining how deep invagination of the pit leads to fission.

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Introduction

Clathrin-mediated endocytosis initiates with the recruitment of clathrin coat components to the plasma membrane, which

is followed by the invagination of the membrane to form a clathrin-coated pit. It is completed by the fission reaction in which dynamin GTPase plays a key role (Slepnev and De Camilli, 2000; Takei and Haucke, 2001). Dynamin polymerizes into rings at the neck of clathrin-coated pits, but its precise mechanism of action in fission remains unclear. *In vitro* studies have shown that dynamin binds to lipids, and it can deform lipid bilayers into narrow tubules and fragment them in a GTP-hydrolysis-dependent way. Thus, one model proposes that dynamin acts as a mechanoenzyme, that is, by constricting the vesicle neck via a conformational change coupled to its catalytic action (Takei *et al*, 1995; Sweitzer and Hinshaw, 1998; Marks *et al*, 2001; Chen *et al*, 2004). Another model proposes that GTP-bound dynamin functions by activating a still unidentified downstream effector (Sever *et al*, 1999; Song and Schmid, 2003). The GTPase module of dynamin is located at the N-terminus of the protein. This domain is followed by the evolutionary conserved middle domain, a pleckstrin homology (PH) domain, and the C-terminal proline/arginine-rich domain (PRD). The region between the PH domain and the PRD is referred to as the GTPase effector domain (GED), because it binds the GTPase module of adjacent dynamin molecules within dynamin polymers and may function as a GTPase activating protein (GAP) (Muhlberg *et al*, 1997). The PH domain binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂), while the PRD binds a variety of src-homology 3 (SH3) domain-containing proteins. These interactions also have been reported to stimulate dynamin GTPase activity (Gout *et al*, 1993; Zheng *et al*, 1996; Barylko *et al*, 1998). Dynamin 1 is the best-characterized dynamin isoform and is enriched at synapses.

Amphiphysin, which is present in brain primarily as a dimer of two similar isoforms, amphiphysin 1 and 2, is a major binding partner of dynamin 1 in the vertebrate nervous system. Amphiphysin binds the PRD of dynamin via a C-terminal SH3 domain and co-assembles with dynamin into rings either in solution or on lipid tubules (Takei *et al*, 1999). This interaction is expected to be physiologically important in nerve terminals, because acute disruption of amphiphysin by peptide microinjection leads to a block of synaptic vesicle endocytosis (Shupliakov *et al*, 1997). The N-terminal portion of amphiphysin contains a BAR (BIN/amphiphysin/Rvs) domain that mediates homo- and heterodimerization (Wigge *et al*, 1997; Ramjaun *et al*, 1999). Via this domain, amphiphysin, like dynamin, binds to and tubulates liposomes (Takei *et al*, 1999), and can also generate narrow plasma membrane tubules in living cells when overexpressed (Peter *et al*, 2004).

The structure of the BAR domain of amphiphysin was recently determined by crystallographic analysis and found to represent an evolutionary conserved protein module present in a variety of proteins (Habermann, 2004; Peter *et al*, 2004). It is represented by a triple helix arranged in an

*Corresponding author. Department of Neuroscience, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. Tel.: +81 86 235 7120; Fax: +81 86 235 7126; E-mail: kohji@md.okayama-u.ac.jp

⁶These authors contributed equally to this paper

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antiparallel dimer to form a crescent-like structure. It was proposed that lipid interactions mediated by the concave portion of this module, in cooperation with an amphipathic N-terminal helix (Farsad *et al*, 2001), may be responsible for its property to curve the bilayer (Peter *et al*, 2004). In addition, lipid-binding studies revealed preferential binding to small liposomes, suggesting that the BAR domain may also function as a curvature sensor, thus facilitating the recruitment of amphiphysin to pre-existing high curvature membranes (Peter *et al*, 2004). Curvature-sensitive properties of proteins may have an important role in orchestrating vesicle formation and fission, with the sequential recruitment and dissociation of cytosolic factor (Bigay *et al*, 2003). The central region of brain amphiphysin 1 and 2 contains binding sites for clathrin and for the clathrin adaptor protein 2 (AP-2) (Ramjaun and McPherson, 1998; Slepnev *et al*, 2000; Miele *et al*, 2004). Via this multiplicity of interactions, which are regulated by an intramolecular binding (Farsad *et al*, 2003), cyclin-dependent kinase 5-dependent phosphorylation (Floyd *et al*, 2001; Tomizawa *et al*, 2003) and calcineurin-dependent dephosphorylation (Bauerfeind *et al*, 1997), amphiphysin is thought to play an important role in coordinating vesicle budding and fission (Farsad *et al*, 2003). Accordingly, defects in synaptic vesicle recycling were demonstrated in amphiphysin 1 knockout mice (Di Paolo *et al*, 2002). In these mice, not only expression of amphiphysin 1 is suppressed, but also the level of amphiphysin 2 is severely reduced in brain, probably due to the stabilizing effect of the amphiphysin heterodimer on the turnover of amphiphysin 2.

Although these studies support the importance of the amphiphysin–dynamin interaction in endocytosis, it remains unclear whether amphiphysin is directly implicated together with dynamin in the fission reaction. The reported lack of significant amphiphysin expression in *Drosophila* neurons (Leventis *et al*, 2001; Razzaq *et al*, 2001; Zelfhof *et al*, 2001) and of a major synaptic vesicle recycling defect in amphiphysin 1 knockout mice raise questions on the importance of this partnership. However, other proteins with similar domain structure and enriched at synapses, for example, endophilin and intersectin/pacsin, may functionally replace amphiphysin (Simpson *et al*, 1999; Modregger *et al*, 2000; Qualmann and Kelly, 2000; Farsad *et al*, 2001). In this study, we demonstrate the importance of amphiphysin for the vesicle-generating properties of dynamin 1 within the context of mouse brain cytosol *in vitro*. We also show a striking stimulatory effect of amphiphysin 1 on the GTPase activity of dynamin in the presence of liposomes, which is dependent on the size of liposomes.

Results

Amphiphysin potentiates vesicle formation by dynamin

Dynamin-dependent vesicle formation can be reconstituted *in vitro* by incubation of large unilamellar liposomes with brain cytosol in the presence of nucleotides, and monitored quantitatively by dynamic light scattering (DLS) (Kinuta *et al*, 2002). To establish the importance of amphiphysin in vesicle formation, we compared brain cytosol from wild-type and amphiphysin 1 knockout mice in this assay. The brain cytosol of the knockout mice is also nearly completely devoid of amphiphysin 2. When incubated with large unilamellar liposomes (1779.5 ± 461.7 nm in diameter) (Figure 1A), wild-

type cytosol induced a massive formation of small vesicles (112.3 ± 19.7 nm in diameter) (Figure 1B), while knockout mice brain cytosol was very inefficient in this process (Figure 1C). At the end of the incubation, small vesicles represented $82.9 \pm 11.7\%$ of the total vesicle number in the wild-type cytosol sample, but only $6.8 \pm 5.5\%$ in the knockout cytosol sample (Figure 1B and C). Addition of purified amphiphysin 1 ($0.5 \mu\text{g/ml}$) to the knockout cytosol rescued vesicle formation, with small vesicles (176.8 ± 33.9 nm in diameter) representing $82.1 \pm 4.3\%$ of the total vesicles at the end of the reaction (Figure 1E). No vesicle formation was observed when liposomes were incubated with amphiphysin 1 alone (Figure 1D). Results of these assays are summarized in Figure 1F.

We next examined the effect of purified amphiphysin 1 on vesicle formation by purified dynamin. Large unilamellar liposomes were incubated with dynamin alone or a mixture of dynamin plus amphiphysin 1 in the presence of GTP. At the end of the reaction, small vesicles represented $15.7 \pm 12.7\%$ of the total vesicles (Figure 2B and D) in the sample containing dynamin 1 alone, and $71.1 \pm 4.5\%$ of the total in the samples containing both proteins (Figure 2C and D), thus indicating a potent action of amphiphysin 1 on the vesicle-generating properties of dynamin. Massive fragmentation of liposomes into small vesicles following incubations with dynamin and amphiphysin 1 was confirmed by negative staining electron microscopy (EM) (Figure 2E).

Stimulation of dynamin GTPase activity by amphiphysin is affected by liposome size

To gain some insight into the mechanisms through which amphiphysin 1 stimulates dynamin-dependent vesicle formation, we examined whether amphiphysin 1 enhanced dynamin GTPase activity. Addition of increasing amount of amphiphysin 1 to a reaction mixture containing dynamin, [$\gamma\text{-}^{32}\text{P}$]GTP and large unilamellar liposomes (1779.5 ± 461.7 nm in diameter) composed of a brain lipid extract (Folch fraction type 1, FF) (74%, w/w), cholesterol (20%) and PtdIns(4,5) P_2 (6%) produced a prominent increase in the GTPase activity. The activity was maximal when the molar ratio of dynamin to amphiphysin 1 was in the 1:1–2 range (Figure 3A). Such effect may be explained in part by enhanced recruitment of dynamin, because amphiphysin also increased the recruitment of dynamin to large liposomes (approximately two-fold) (Figure 3D).

Since small liposomes were often utilized in previous studies on dynamin GTPase activity (Tuma *et al*, 1993; Barylko *et al*, 1998), we also tested small liposomes prepared by sonication (80.7 ± 10.8 nm in diameter) in the GTPase assay. In the absence of amphiphysin, the GTPase activity of dynamin was much higher with small liposomes than with large liposomes, possibly due to a more efficient oligomerization of dynamin on small liposomes. Surprisingly, addition of increasing amount of amphiphysin 1 led to a drastic decrease in the GTPase activity in the presence of the small liposomes. The molar ratio that generated maximal stimulation in the presence of large liposomes produced a 75% inhibition under these conditions (Figure 3B). A similar inhibitory effect was observed also with small liposomes prepared by extrusion (143.8 ± 31.7 nm in diameter) (Figure 3C), ruling out a possible effect of sonication on the properties of the bilayer. Thus, the effect of amphiphysin 1 on

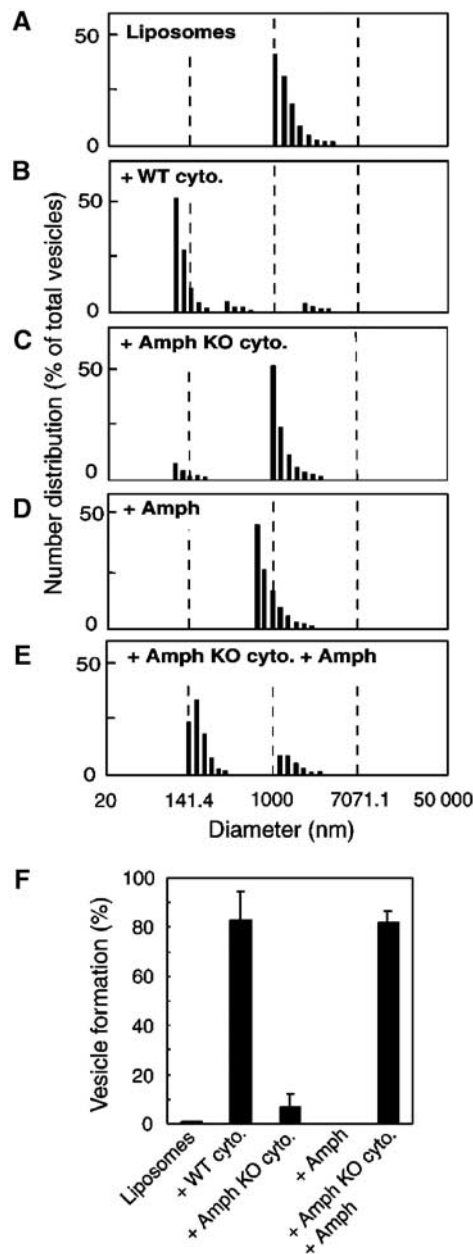


Figure 1 Requirement of amphiphysin 1 for vesicle formation by brain cytosol. (A–E) Vesicle formation quantified by the DLS assay. Data represented are the relative distribution in numbers of formed vesicles. Large unilamellar liposomes were incubated in the presence of 200 μ M GTP and 2 mM ATP under the conditions indicated. (F) Percentage of small vesicles formed in each incubation condition. Vesicles smaller than 200 nm in diameter were defined as small vesicles. Values represent the mean \pm s.d. for more than three independent measurements. Amph = amphiphysin 1; Amph KO cyto. = amphiphysin knockout mice brain cytosol; WT cyto. = wild-type mice brain cytosol.

dynamin GTPase activity is strongly affected by the size of the liposomes.

An attractive possibility is that the stimulation of dynamin GTPase activity is related to the property of amphiphysin and dynamin to co-oligomerize into ring structures around membrane tubules. While large liposomes allow massive tubulation (Takei *et al*, 1999), the size of the small liposomes may be incompatible with tubulation and constriction, explaining the different results obtained with large and small liposomes.

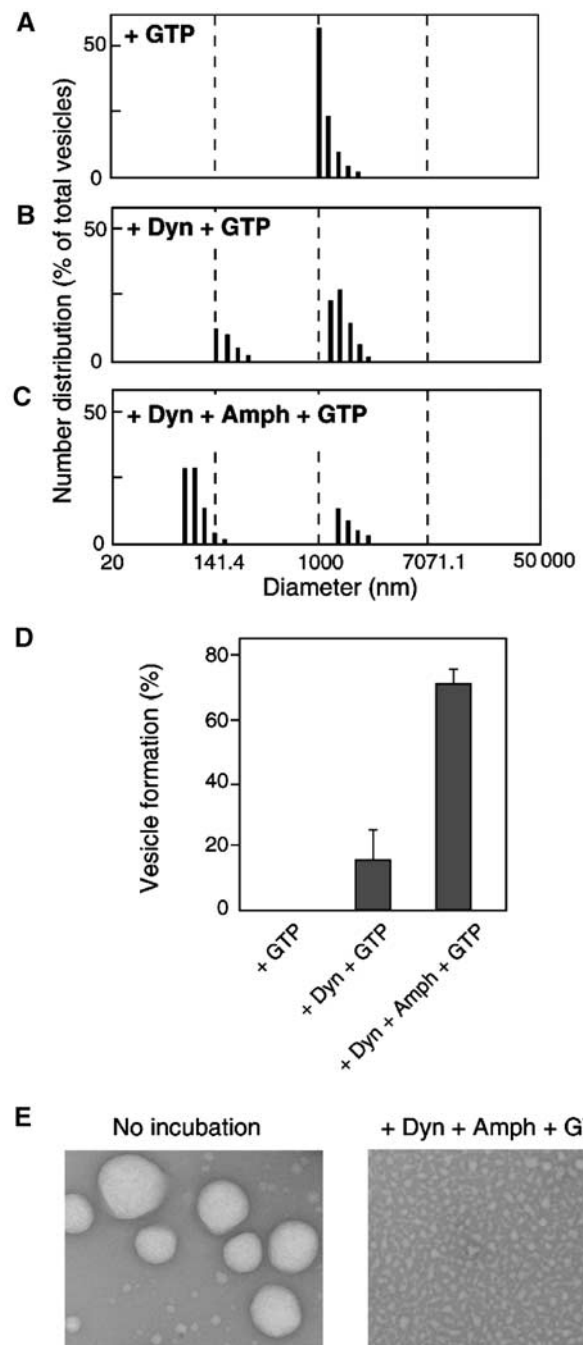


Figure 2 Quantitative analysis of vesicle formation from liposomes by dynamin and amphiphysin 1. (A–C) Vesicle formation by purified proteins was quantified by DLS assay, and the relative number distribution was represented. Large unilamellar liposomes were incubated in the presence of 1 mM GTP under the conditions indicated. The ratio of dynamin (Dyn) to amphiphysin 1 (Amph) was 1:2.67 (mol/mol), a ratio of 1:2 (w/w). (D) Percentage of small vesicles formed in each incubation condition. Values represent the mean \pm s.d. for more than three measurements. (E) Negative-staining EM of large unilamellar liposomes before incubation (left), and vesicles formed by incubation with dynamin and amphiphysin 1 (right). Bar = 100 nm.

Stimulatory action of amphiphysin is affected by lipid component

If the effect of amphiphysin on dynamin GTPase activity is linked to its property to co-oligomerize with dynamin on tubular membranes, the effect should be strongly dependent

on the presence of liposomes. Therefore, we examined whether amphiphysin stimulates dynamin GTPase activity in the presence or absence of liposomes. In the absence of liposomes, the GTPase activity of dynamin was low, and amphiphysin 1 stimulated this activity only slightly, approxi-

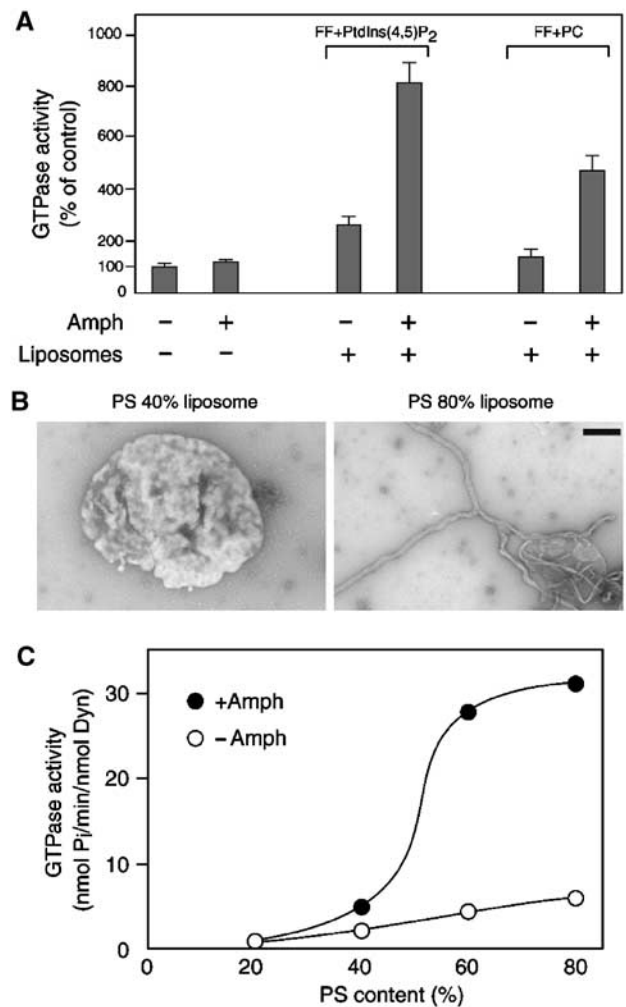
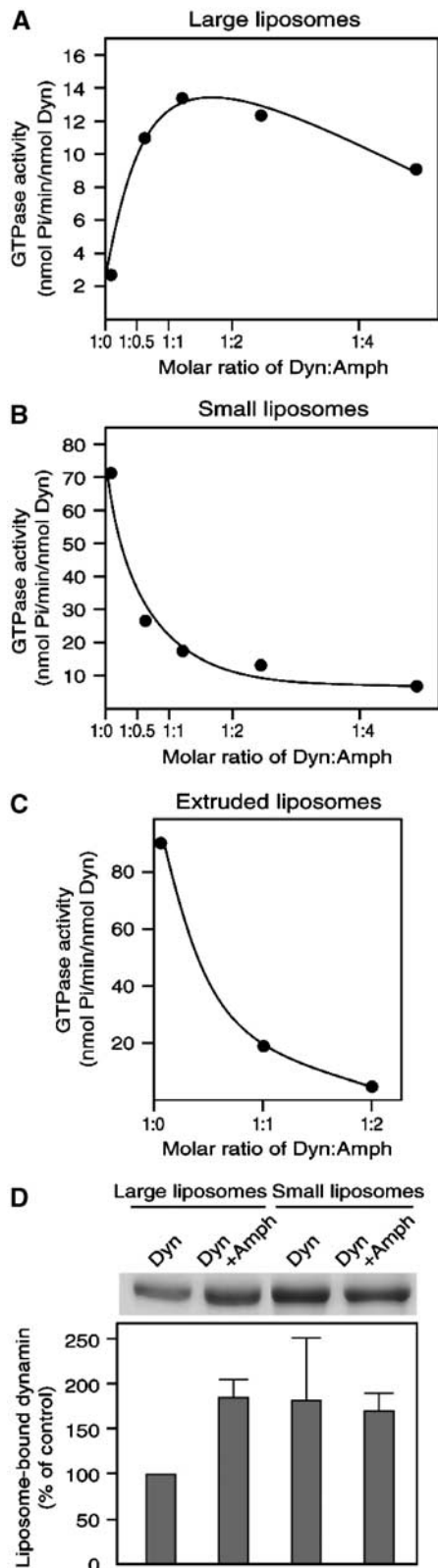


Figure 4 Effect of the lipid composition on stimulation of dynamin GTPase activity by amphiphysin 1. (A) Dynamin GTPase activity measured with or without amphiphysin 1, and in the absence or presence of liposomes. The lipid composition of FF + PtdIns(4,5)P₂ and FF + PC liposomes are cholesterol/FF/PtdIns(4,5)P₂ = 20:74:6% (w/w) and cholesterol/FF/PC = 20:74:6% (w/w), respectively. Results were normalized to the average of control value (1.06 nmol Pi/min/nmol Dyn). (B) Negative-staining electron micrographs of liposomes incubated with amphiphysin 1. Left, a single liposome containing 40% PS (cholesterol/PS/PC = 20:40:40%, w/w) generated irregular surface, but little tubulation was observed. Right, a single liposome containing 80% PS (cholesterol/PS = 20:80%, w/w) formed massive tubules. Bar = 500 nm. (C) Dynamin GTPase activity measured with (closed circles) or without (open circles) amphiphysin 1, in the presence of liposomes containing various amounts of PS, PC and 20% cholesterol. Data represent the mean value of triplicate measurements.

Figure 3 Stimulation of dynamin GTPase activity by amphiphysin 1. Dynamin (0.2 μ M) and increasing amount of amphiphysin 1 were incubated either with 2 μ g of large unilamellar liposomes (A) or with 2 μ g of sonicated small liposomes (B), in the presence of 1 mM GTP containing [γ -³²P]GTP (0.8 μ Ci). After incubation, ³²P_i released from the radiolabeled GTP was measured. Data represent the mean value of triplicate measurements. (C) GTPase activity assayed using extruded small liposomes. (D) Recruitment of dynamin to large unilamellar liposomes (left two lanes) or to sonicated small liposomes (right two lanes) in the presence or absence of amphiphysin 1. Results shown are the average of three assays \pm s.d.

mately 1.2-fold (compare lanes 1 and 2 in Figure 4A). The presence of large unilamellar liposomes (FF (74%, w/w), cholesterol (20%) and PtdIns(4,5)P₂ (6%)) produced a 2.6-fold increase in dynamin GTPase activity (compare lanes 1 and 3 in Figure 4A), and the additional presence of amphiphysin 1 produced a further 3.1-fold stimulation (compare lanes 3 and 4 in Figure 4A). Replacement of PtdIns(4,5)P₂ with L- α -phosphatidylcholine (PC) in the liposomes reduced the stimulation produced by the liposomes (compare lanes 3 and 5 in Figure 4A), consistent with the known role of PtdIns(4,5)P₂ in dynamin recruitment and activation. However, the stimulatory effect of amphiphysin 1 on the GTPase activity of dynamin was nearly unaffected (approximately 3.4-fold) (compare lanes 5 and 6 in Figure 4A).

Another acidic phospholipid was tested. L- α -Phosphatidylserine (PS) was reported to support the binding and polymerization of both dynamin and amphiphysin 1 and to stimulate the GTPase activity of dynamin (Figure 4B, Tuma *et al*, 1993; Sweitzer and Hinshaw, 1998; Takei *et al*, 1999). Thus, liposomes containing variable amounts of PS (from 20 to 80%) were used in the tubulation and GTPase assays. Amphiphysin 1 tubulated very efficiently liposomes containing the higher concentrations of PS (Figure 4B, Takei *et al*, 1999). The GTPase activity of dynamin in the absence of amphiphysin 1 increased linearly in parallel with the concentration of PS (Figure 4C, open circles). The presence of amphiphysin 1 produced an additional powerful stimulation of the GTPase activity, which was highly synergistic as the amount of PS was increased. The sigmoidal shape of the stimulation curve indicated a strong positive cooperativity when the amount of PS was between 40 and 60% (Figure 4C, closed circles), possibly reflecting gathering of PS and preferential assembly of dynamin–amphiphysin polymers on PS-rich microdomains.

Both BAR domain and SH3 domain of amphiphysin 1 are required for the stimulation of dynamin GTPase activity

The effect of truncated amphiphysin 1 molecules (Figure 5A) was investigated to identify the domains of the protein responsible for its stimulatory action on the catalytic activity of dynamin. These fragments were tested in the presence of large liposomes composed of FF (74%), cholesterol (20%) and PtdIns(4,5)P₂ (6%). Under this assay condition, full-length amphiphysin 1 stimulated the GTPase activity about three-fold (Figure 5B, see also lanes 3 and 4 in Figure 4A). A roughly similar stimulation was produced by Amph 1–226, which comprises the BAR domain only, and by Amph 1–306, which contains an additional flanking region at the C-terminal side of this domain. Strikingly, Amph Δ 248–601, which contains both BAR and SH3 domains, but lacks the central region, produced a 26-fold stimulation of the GTPase activity. As the proline-rich stretch binds intramolecularly to the SH3 domain and regulates negatively the interaction of amphiphysin with dynamin (Farsad *et al*, 2003), we also tested Amph Δ 248–315, a mutant lacking the proline-rich stretch. This mutant stimulated the GTPase activity about three-fold over the value obtained with full-length amphiphysin 1. Amph 545–695 alone, corresponding to the C-terminal SH3 domain, had no effect (Figure 5B). Furthermore, stimulation of the GTPase activity by the BAR domain was not enhanced by the presence of the free SH3 domain (data not shown). These results suggested that presence of both the BAR

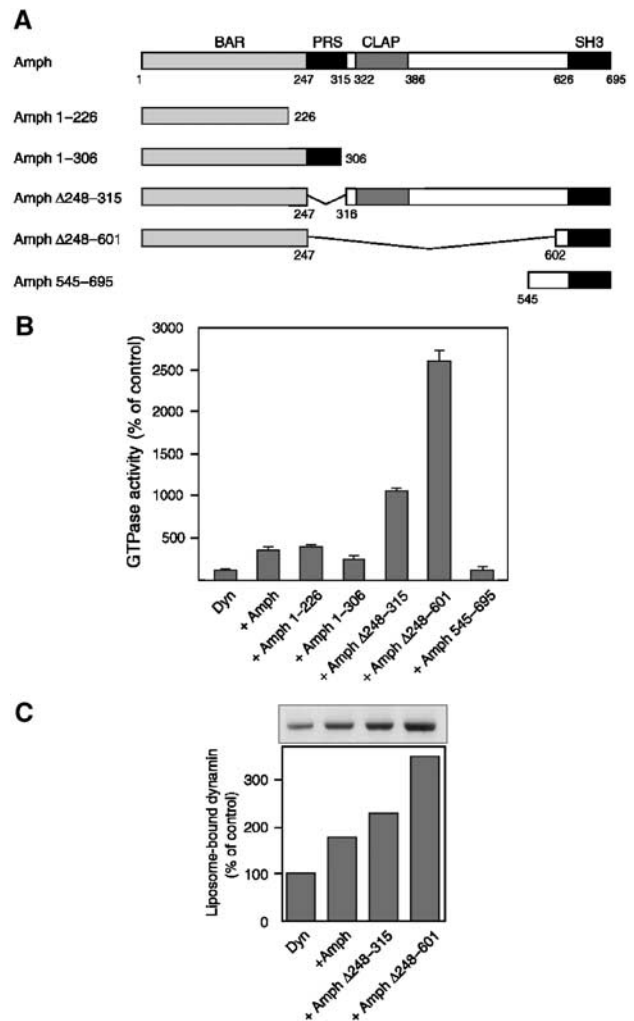


Figure 5 Amphiphysin 1 domains required for the stimulation of dynamin GTPase activity. (A) Domain structure of amphiphysin 1 constructs used for assays. Numbers indicate amino-acid residues of full-length amphiphysin 1. (B) Dynamin GTPase activity in the presence of each construct and of large unilamellar liposomes containing 74% FF, 20% cholesterol and 6% PtdIns(4,5)P₂. Dyn:Amph = 1:2 (mol/mol). Data represent the mean value of triplicate measurements. Results were normalized to the average of control value (2.86 nmol Pi/min/nmol Dyn). (C) Dynamin binding to large liposomes in the presence of amphiphysin or truncated mutants was checked by the sedimentation assay. BAR = BIN/amphiphysin/Rvs; PRS = proline-rich stretch; CLAP = clathrin-AP-2-binding domain; SH3 = src-homology 3.

domain and the SH3 domain in a single molecule was critical for the stimulation of dynamin GTPase activity.

The observed effects of the amphiphysin deletion constructs on the GTPase activity of dynamin correlated qualitatively with their property to enhance dynamin recruitment to large liposomes, with Amph Δ 248–601 producing a more than three-fold increase in recruitment (Figure 5C).

Both BAR domain and SH3 domain of amphiphysin 1 are sufficient for the formation of rings

The incubation of dynamin and amphiphysin 1 in physiological buffer results in the formation of ring structures, similar in size to dynamin-containing collars of endocytic pits (Takei *et al*, 1999). These collars are thought to represent a transient

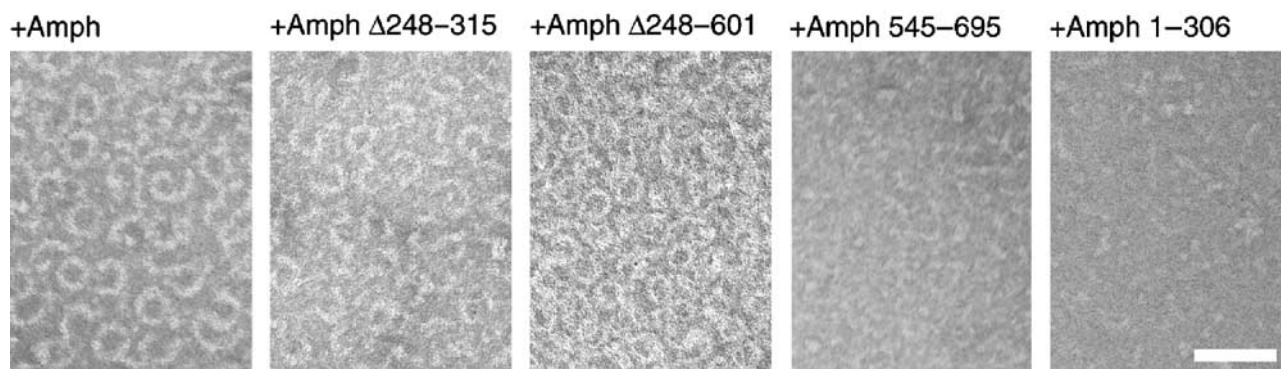


Figure 6 Negative staining EM of reaction mixtures containing dynamin and each construct indicated. Note the massive ring formation with full-length amphiphysin 1, Amph Δ 248–315 or with Amph Δ 248–601. Bar = 100 nm.

intermediate in the fission reaction. We used negative staining EM to determine whether these rings were present following incubation of dynamin with the truncated amphiphysins. Rings were observed after incubation of dynamin with full-length amphiphysin 1, with Amph Δ 248–315, or with Amph Δ 248–601, but not with the other amphiphysin 1 fragment (Figure 6). The diameter of rings with Amph Δ 248–601 (41.2 ± 3.0 nm of outer diameter and 22.3 ± 2.6 nm of inner diameter) was slightly shorter than that of rings with amphiphysin 1 (outer diameter 51.7 ± 4.3 nm and inner diameter 27.5 ± 3.3 nm).

Discussion

We provide strong evidence for the physiological role of amphiphysin in the regulation of dynamin function and new insights concerning the mechanisms through which amphiphysin achieves this effect. Several previous reports, including studies in living cells and cell-free studies, had suggested an important partnership of amphiphysin and dynamin in the fission reaction of endocytic vesicles, in particular in the fission of recycling synaptic vesicles at synapses (Shupliakov *et al*, 1997; Takei *et al*, 1999). Yet, the importance of this interaction has been challenged by studies in *Drosophila*, where amphiphysin is expressed primarily outside the nervous system (Leventis *et al*, 2001; Razzaq *et al*, 2001; Zehhof *et al*, 2001), and by the relatively mild neurotransmission defects observed in amphiphysin 1 knockout mice (Di Paolo *et al*, 2002), which lack both amphiphysin 1 and 2 in the neuronal cytoplasm. In contrast with these findings, we have now found that the property of brain cytosol to vesiculate large liposomes is drastically impaired in the cytosol of amphiphysin 1 knockout brain (Figure 1). Since this property was previously shown to be mediated mainly by dynamin (Takei *et al*, 1998; Kinuta *et al*, 2002), our findings point to a role of amphiphysin in the regulation of dynamin. Addition of amphiphysin 1 is sufficient to rescue the defect of the knockout brain cytosol, indicating that in this assay the amphiphysin 1 homodimer and the amphiphysin 1/2 heterodimer have similar properties. The discrepancy between these *in vitro* results and *in vivo* data may reflect the compensatory action *in vivo* of other BAR domain-containing proteins (Peter *et al*, 2004), such as endophilin and syndapin/pacsin, that have a domain structure similar to amphiphysin (Qualmann and Kelly, 2000; Habermann, 2004; Peter *et al*, 2004) and are also present at

synapses. The importance of amphiphysin relative to other proteins with overlapping function may be greater in *in vitro* assays than *in situ* because of differential compartmentalization and/or regulation in living nerve terminals. Furthermore, yet unidentified molecules present in cellular membranes, and absent in our cell-free assays, may compensate for the absence of amphiphysin.

We have found that the property of amphiphysin to stimulate the GTPase activity of dynamin is critically influenced by the presence of liposomes and that this effect, in turn, is strongly affected by the composition and size of liposomes (Figures 3 and 4). The presence of acidic phospholipids is required, as expected. Amphiphysin stimulated the GTPase activity of dynamin and its binding on large unilamellar liposomes. Large liposomes, which can be evaginated to narrow tubules, may allow dynamin and amphiphysin to co-oligomerize into a stack of rings, and the co-oligomers bound to lipids may represent the configuration with the maximal GTPase activity. In contrast, amphiphysin inhibited the GTPase activity, with little change in the dynamin recruitment in the presence of small liposomes. These results suggest that amphiphysin–dynamin rings may not assemble on small liposomes, whose size may prevent the formation of narrow tubular membranes. In this context, amphiphysin may in fact perturb dynamin polymerization. The effect of amphiphysin 1 on the GTPase activity of dynamin was analyzed previously (Wigge *et al*, 1997; Takei *et al*, 1999). We have reported that amphiphysin 1 stimulates dynamin GTPase activity in the absence of liposomes, and that in the presence of liposomes addition of amphiphysin 1 did not further stimulate this activity (Takei *et al*, 1999). A stimulatory effect of amphiphysin 1 and 2 on the catalytic activity of dynamin was also reported by another group (Wigge *et al*, 1997). As clearly indicated in this study, the activity of dynamin is dependent on the experimental conditions, thus explaining the inconsistency between the results reported here and previous studies.

As for the molecular mechanisms by which amphiphysin stimulates dynamin GTPase activity, some clues are provided by experiments using amphiphysin deletion mutants (Figures 5 and 6). Surprisingly, BAR domain-containing constructs that miss the dynamin-binding domain (the SH3 domain) can stimulate the GTPase activity of dynamin in the presence of liposomes (Figure 5B). Most likely, the powerful liposome-tubulating activity of these constructs enhances the GTPase activity by providing a template for dynamin polymerization.

Although dynamin alone can tubulate liposomes, it may assemble more efficiently on previously formed lipid tubules. For example, dynamin has been reported to assemble efficiently on preformed lipid tubules (Stowell *et al*, 1999) and microtubules (Shpetner and Vallee, 1989). The amphiphysin construct missing the central region, or a portion of it comprising the proline-rich stretch, stimulated dynamin GTPase activity more potently than full-length amphiphysin. This is consistent with the occurrence of an autoinhibitory intramolecular interaction in amphiphysin (Farsad *et al*, 2003). The SH3 domain alone had no effect on the GTPase activity of dynamin in the presence of liposomes. This indicates that the importance of the SH3 domain relates to its ability to recruit dynamin to the membrane via the BAR domain. Collectively, these results provide new support for the hypothesis that amphiphysin functions together with dynamin in fission (Takei *et al*, 1999), both by helping the generation of membrane curvature and by facilitating the recruitment and oligomerization of dynamin.

The precise orientation of dynamin and amphiphysin in the rings remains to be elucidated. The amphiphysin–dynamin rings are similar in diameter to the rings formed by dynamin alone. This observation suggests a model in which amphiphysin and dynamin are arranged side by side along the ring. Such arrangement would be consistent with the greater thickness of the hybrid amphiphysin–dynamin ring (Takei *et al*, 1999). Cryo-EM studies have shown that dynamin rings generated by purified dynamin 1 around tubular liposomes comprise about 13–15 dynamin dimers (Zhang and Hinshaw, 2001; Chen *et al*, 2004). Considering the curvature of the BAR domain, a ring could not accommodate an equivalent number of crescent-shaped BAR domains (Peter *et al*, 2004). Thus, BAR domains may be arranged obliquely, with their positively charged edges juxtaposed to the negatively charged middle portion of the adjacent BAR domain (Figure 7).

Irrespective of the precise stoichiometry of amphiphysin and dynamin around tubular lipid templates, these results add new strong support to a model implicating amphiphysin in the regulation of clathrin-mediated endocytosis (Figure 7).

At early stages of clathrin-coated pit formation, the curvature-generating properties of amphiphysin may help maturation of the bud into a deeply invaginated pit. In addition, via a positive feedback mechanism, preference of its BAR domain for a curved membrane with radius in the size range of bud neck may enhance its concentration at the neck as the coated pit forms. This concentration could facilitate dynamin oligomerization into a ring. Dynamin recruitment may be further facilitated by the binding of the central domain of amphiphysin to clathrin and AP-2, which results in the release of the SH3 domain from the autoinhibitory intramolecular interaction (Farsad *et al*, 2003). In conclusion, our results suggest a model in which narrowing of the stalk of an endocytic pits is coupled to activation of dynamin GTPase activity, thus explaining how the deep invagination of the pit may lead to fission.

Materials and methods

Materials

Bovine brain lipid extract (Folch fraction type I, FF) and PtdIns(4,5)P₂ from bovine brain were purchased from Sigma (St Louis, MO). PS and PC were from Avanti Polar Lipids (Alabaster, AL). Glutathione sepharose 4B beads, pGEX-6P vector, PreScission protease and thrombin were from Amersham Bioscience (Piscataway, NJ). [γ -³²P]GTP (5 kCi/mol) was from the Institute of Isotopes Co. Ltd (Hungary).

Preparation of brain cytosol and dynamin 1

Brain cytosol from wild-type mice and amphiphysin 1 knockout mice (Di Paolo *et al*, 2002) was prepared essentially by the previous method and used (Kinuta *et al*, 2002). Dynamin 1 was purified from bovine brains by the method of Liu *et al* (1994). The dynamin solution was concentrated using Centrplus YM50 (Millipore, MA), and stored at –80°C. The protein solution (0.6 mg/ml protein) was thawed at 37°C before use.

Preparation of amphiphysin 1 and its truncation constructs

The cDNAs encoding full-length human amphiphysin 1 and its truncation constructs were prepared by PCR amplification using specific primers. Full-length amphiphysin 1, Amph 1–226 and 1–306 were subcloned into pGEX-6P vector as *Bam*HI–*Eco*RI fragments. To prepare Amph Δ 248–315, Amph 1–247aa was

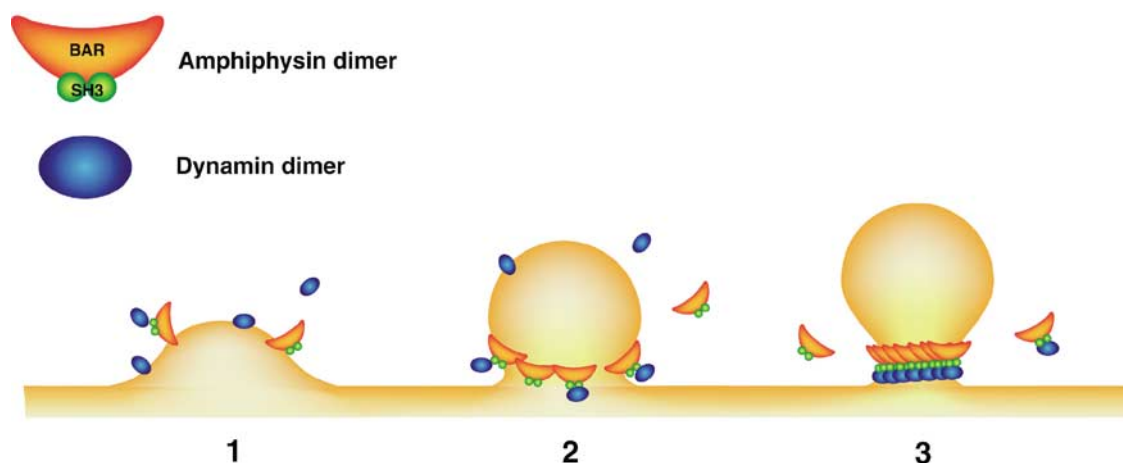


Figure 7 Possible function of amphiphysin in the fission process of endocytic pit. (1) Amphiphysin is recruited to the membrane initiating invagination either via its BAR domain or via clathrin/AP-2-binding domain. (2) The BAR domain may help generating the bud neck of the appropriate dimensions. (3) As the pit has been matured, amphiphysin could be more efficiently recruited to the neck due to its curvature-sensing properties. Dynamin also assembles to form a ring at the bud neck, and its GTPase is fully activated so that vesicle fission is achieved. Coat components are omitted.

subcloned into pGEX-6P vector as a *Bam*HI–*Eco*RI fragment. *Eco*RI–*Eco*RI fragment containing Amph 316–695aa was then inserted into the *Eco*RI site. As for Amph Δ 248–601, BAR domain was subcloned into pGEX-6P vector as a *Bam*HI–*Nco*I fragment, and inserted at the *Bam*HI–*Nco*I site of full-length amphiphysin 1 plasmid described above. The SH3 domain was subcloned into pGEX-2T vector. The nucleotide sequences of the constructs were verified using a DNA sequence analyzer. The expression of GST-fusion proteins was induced by 0.1 mM isopropyl-1-thio- β -D-galactopyranoside at 37°C for 3–6 h in LB medium supplemented with 100 μ g/ml ampicillin at A_{600} = 0.8. The purification of GST-fusion proteins was performed as described previously (Slepnev *et al*, 2000), and the cleavage of the GST with PreScission protease was carried out according to the manufacturer's instruction. Finally, it was purified on MonoQ column equilibrated in 20 mM Tris–HCl (pH 7.7) and 0.2 M NaCl. The protein solution (1 mg/ml protein) was stored at –80°C, and thawed at 37°C before use.

Preparation of liposomes

Large unilamellar liposomes in 0.3 M sucrose (1 mg/ml) were prepared as described (Takei *et al*, 2001). The size of large unilamellar liposomes measured using DLS was 1779.5 ± 461.7 nm in diameter. Small liposomes were made from large unilamellar liposomes by sonication (80.7 ± 10.8 nm in diameter). Extruded liposomes were prepared by extrusion of large liposomes through polycarbonate membranes with pores of 100 nm for five times (143.8 ± 31.7 nm in diameter).

Formation of small vesicles from liposomes

Large unilamellar liposomes composed of 80% (w/w) FF and 20% cholesterol in 0.3 M sucrose (1 mg/ml) were prepared as described previously (Takei *et al*, 1999). Vesicle formation was performed by the method of Kinuta *et al* (2002), with minor modification. A reaction mixture (500 μ l) containing cytosolic buffer (25 mM Hepes–KOH (pH 7.2), 25 mM KCl, 2.5 mM magnesium acetate, 100 mM potassium glutamate), 100 μ g of liposomes, brain cytosol from either wild-type or amphiphysin knockout mice (500 μ g/ml protein), 2 mM ATP and 200 μ M GTP was incubated at 37°C for 15 min. As for vesicle formation by purified proteins, 25 μ g dynamin 1 and 50 μ g of amphiphysin 1 were made to react. The size and relative numbers in each size of the formed vesicles were measured by DLS assay (Kinuta *et al*, 2002; Tomizawa *et al*, 2003). Formed vesicles smaller than 200 nm in diameter were defined as small vesicles.

Liposome sedimentation assay

For liposome sedimentation assay, *in vitro* incubation was performed under the similar protein lipid concentration as used for GTPase assay. Dynamin (0.2 μ M) and amphiphysin (0.4 μ M) or

amphiphysin truncation construct (0.4 μ M) were incubated with 10 μ g of liposomes, composed of 74% (w/w) FF, 20% cholesterol and 6% PtdIns(4,5) P_2 , in the cytosolic buffer at 37°C for 15 min. The reaction mixture was then centrifuged at 20 600 g for 10 min. The proteins in the pellet were analyzed by SDS–PAGE, subsequently checked by Coomassie blue staining. The quantification of binding protein was performed by scanning the gel and analyzing the scanned images with the NIH image program.

Dynamin GTPase assay

GTPase activity was measured under similar condition as DLS assay, with little modification of liposome composition, 74% (w/w) FF, 20% cholesterol and 6% PtdIns(4,5) P_2 . Other lipid compositions of liposomes are described in figure legends. Small liposomes were prepared by sonication of the large unilamellar liposomes. Assay was performed essentially as described (Barylko *et al*, 2001). A reaction mixture (100 μ l) containing the cytosolic buffer, 0.2 μ M dynamin, 2 μ g liposomes and either amphiphysin 1 or 0.4 μ M truncated amphiphysins was incubated at 37°C for 5 min. Reactions were initiated by the addition of [γ - 32 P]GTP. After incubation, 32 P $_i$ from [γ - 32 P]GTP was extracted with benzene in the presence of silicotungstic acid and (NH $_4$) $_6$ Mo $_7$ O $_{24}$ ·4H $_2$ O, and measured by a liquid scintillation counter using Packard 2260XL (Hewlett Packard, Houston, TX) at the Department of Radiation Research, Shikata Laboratory Advanced Science Research Center Okayama University.

Electron microscopy (EM)

To test the tubulation of large unilamellar liposomes, 1.5 μ g of amphiphysin 1 was incubated with 4 μ g of liposomes at the same condition. To test the formation of dynamin–amphiphysin ring structures, mixtures of dynamin 1 and either amphiphysin 1 or truncation constructs (molar ratio = 1:1.5) in the cytosolic buffer were incubated at 37°C for 15 min. The reaction mixture is adsorbed to carbon-coated EM grids that had been glow-discharged just prior to use, and negatively stained with 2% aqueous uranyl acetate. The grids were observed under a Hitachi H-7100 transmission electron microscope (Japan) at the Central Research Laboratory at Okayama University Medical School.

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